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Paw Inflammation Model in Dogs for Preclinical Pharmacokinetic/Pharmacodynamic Investigations of Nonsteroidal Anti-Inflammatory Drugs

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ABSTRACT

The goal of the present study was to develop and validate a new canine model of inflammation. The motivation was to make available a scientifically appropriate and ethically acceptable model to conduct pharmacokinetic/pharmacodynamic investigations for testing nonsteroidal anti-inflammatory drugs in dogs. A kaolin-induced paw inflammation model previously developed in cats was adapted to the dog. The paw inflammation developed within a few hours, reached maximum values 24 h and up to 3 days after kaolin administration, and then progressively resolved over 2 months. Five end points of clinical interest (body temperature, creeping time under a tunnel, paw withdrawal latency to a standardized thermal stimulus, lameness score, and vertical force developed during walking on a force plate) were measured regularly over the next 24 h and beyond

to characterize the time development of the inflammation either in control conditions (placebo period) or after the administration of meloxicam (test period) according to a crossover design. Pharmacodynamic data were modeled using an indirect response pharmacokinetic/pharmacodynamic model. This model described three effects of meloxicam, namely, classic anti-inflammatory, analgesic, and antipyretic effects. The mean plasma meloxicam IC_{50} values were 210 ng/ml for the antipyretic effect, 390 ng/ml for the analgesic effect, and 546 ng/ml for the vertical force exerted by the paw on the ground as measured by force plates. These *in vivo* IC_{50} values require approximately 80 (antipyretic effect) to 90% (all other effects) cyclooxygenase-2 inhibition as calculated *ex vivo* whole-blood assay data.

Introduction

Few preclinical studies have attempted to model plasma concentration-time profiles with the time course of nonsteroidal anti-inflammatory drug (NSAID) effects in small carnivores (Toutain et al., 2001; Giraudel et al., 2009). A requisite to carry out a pharmacokinetic/pharmacodynamic (PK/PD) dose determination is the availability of an animal model in which the measured end points are clinically relevant, measurable with good metrological performances, and long enough to prevent (or minimize) confusion between the action of the investigated NSAID and the natural time development of the inflammatory process. Currently, only acute knee joint synovitis using intra-articular injection of sodium

urate crystals has been well described in dogs, and its application to the preclinical evaluation of different NSAIDs is now well documented (Cross et al., 1997). However, this model is of too short in duration (less than 24 h) to allow a PK/PD investigation for drugs having a duration of action of approximately 24 h. Likewise, the carrageenan-induced acute paw inflammation in dogs model proposed by Brooks et al. (1991) was too short in duration for this purpose. This is why we developed a Freund's complete adjuvant osteoarthritis model, resulting in a sustained and relatively stable secondary inflammatory response (Botrel et al., 1994; Toutain et al., 2001). This Freund's model was scientifically attractive, allowing repeatable measurements of relevant clinical end points, but the inflammation was irreversible, and it was mandatory to sacrifice dogs for ethical reasons. Because we are committed to a program of rehabilitating dogs by donating them as companion animals at the end of the trials, we adopted the 3R principles to avoid unnecessary suffering to

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PK/PD, pharmacokinetic/pharmacodynamic; CV, coefficient of variation.

animals [i.e., replace (use alternatives to animal testing whenever possible), reduce (improve existing methods so that fewer laboratory animals are required in an experiment), and refine (improve existing methods so that animals experience as little discomfort and distress as possible)]. The PK/PD approach itself can be considered as a refinement of a dose titration allowing a reduction in the required number of animals to establish a full dosage regimen (dose and dosing interval), but to achieve our ultimate goal of rehabilitating experimental animals, it was necessary to develop and validate a new reversible inflammation model in dogs. Such a model was developed recently in cats (Giraudel et al., 2005a). It consisted of administering kaolin (an inert foreign body) as a phlogistic agent in the paw. The resulting inflammation was sustained between 1 and 3 days after kaolin injection, allowing administration of the NSAID on day 2. The model was found to be suitable for simultaneously studying the analgesic, anti-inflammatory, and antipyretic effects of NSAIDs in cats by measuring different end points such as body temperature or gait scoring (Giraudel et al., 2005a,b). Moreover, because the kaolin was administered extra-articularly, the inflammation progressively vanished with either the physical wasting of the administered kaolin by direct skin exudation or the encystment of the remnant kaolin fraction, and within a few weeks, most animals returned to the control condition without any apparent sequelae.

The objective of the present study was to transfer and validate this reversible inflammation model in dogs, a species used extensively in regulatory studies in both human and veterinary medicine. We selected meloxicam as a test NSAID, because it is a well established NSAID in dogs (Aragon et al., 2007) and also used in man.

Materials and Methods

Animals. Eight healthy Beagle dogs (two females and six males) were selected after clinical examination and biochemical analysis. The body weights and ages of the dogs ranged from 12.5 to 17.5 kg and 1 to 3 years, respectively. Between experimental phases, the dogs were housed in large boxes. During the different phases of the trials, the animals were kept in individual stainless steel cages in a controlled environment. On the days of the measurements, the dogs were placed in their cages at least 2 h before any measurement. Dogs were fed each evening after the last measurements with 250 ± 50 g of commercial dry food (medium; Royal Canin, Aimargues, France). Animal care and conduct of the study were performed according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). The study has been carried out in accordance with the Declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the U.S. National Institutes of Health. The protocol was approved by the Animal Experimentation Ethics Committee of Midi-Pyrenees. The study was performed in compliance with the Principles of Good Clinical Practice (CVMP/VICH/595/98; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004343.pdf) and according to the Guideline for the Conduct of Efficacy Studies for NSAIDs (EMA/CVMP/237/01; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004423.pdf). Because the model is totally reversible without sequelae, all of the dogs that participated in this trial were rehabilitated as companion animals at the end of the study.

Animal Preparation and Induction of Inflammation. Both hind paws were shaved from the toes up to the hock joint, and targets were marked for the pain assessment and paw circumference measures (Fig. 1).

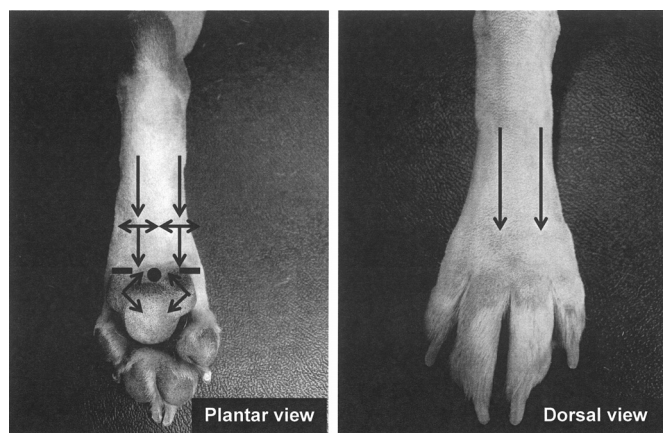


Fig. 1. Cutaneous reference marks on the hind paw of the dog for the circumference (■) and analgesia (●) measurements and view of the different sites of subcutaneous kaolin injection (→).

The preparation of kaolin (hydrated aluminum silicate; Sigma-Aldrich, St. Louis, MO) was done using standardized procedures (Giraudel et al., 2005b). For the induction of inflammation, the dogs were given a general anesthetic with alfaxalone 3 mg/kg (Alfaxan; Vétquinol, Lure, France), and anesthesia was maintained with isoflurane (2% v/v; Aerrane; Baxter, Lessines, Belgium). A local anesthetic was added: approximately 2.5 ml of lidocaine was injected subcutaneously into the paw (Lurocaine; Vétquinol), after which 1.85 ± 0.10 g of kaolin was injected under aseptic conditions subcutaneously at eight different points in the metatarsal pad. The total volume of the kaolin suspension (25% w/w) was 6.4 ml.

Experimental Design. The eight dogs and the investigators were trained regularly for all of the experimental conditions and all of the end point measurements for at least 1 month. This training period guaranteed the eligibility for the trial of the eight selected dogs and the investigators' skills. The trial consisted of two phases. In the first phase, the metrological performances of the different end points were assessed in all of the dogs. Investigated end points were body temperature, paw circumference, time to perform a creeping test under a tunnel, vertical force of the paw on force plates, and thermal pain threshold. Three series of end point measurements were obtained each day and for 5 consecutive days, and the repeatability and reproducibility of the different end points were computed. Lameness scoring was not validated during this first control phase because all of these control measurements were obtained in noninflammatory conditions. Then, during a second phase, a two-period, two-sequence crossover design was carried out. The eight dogs were allocated randomly into four pairs of the same sex and of similar body weight. Dogs of each pair then were allocated into one of two groups (sequences) corresponding to the order of the administration of the test articles (placebo then NSAID or, conversely, NSAID then placebo). The washout period between the two periods of the crossover was 8 weeks. Kaolin inflammation was induced for each dog of a pair and in the two periods (right paw period 1 and left paw period 2). The experiment was blinded to the investigators. The placebo corresponded to a sham meloxicam administration. Meloxicam (Metacam; Boehringer Ingelheim Vetmedica, Ingelheim/Rhein, Germany) was administered by the subcutaneous route 26 h after kaolin inflammation induction. The dose of meloxicam administered was the marketed dose for dogs (0.2 mg/kg). Blood samples were obtained from the jugular vein by direct puncture at time 0 (control) and then 5, 15, 30, and 60 min and, 2, 4, 6, 9, 12, 21, 48, and 72 h after the test article administration (meloxicam or placebo). Before kaolin administration, the PD end points were measured twice the same day and then twice at 22 and 24 h after the induction of the kaolin inflammation. Subsequently, the PD end points were measured at 30 min, 2, 25, 4, 6, 9, 12, 15, 18, 21, 30, and 48 h, and 3, 4, 5, 6, 7, 10, 14, 17, 21, and 24 days after test article administration (i.e., placebo or

TABLE 1

Numerical rating scale for the evaluation of lameness in the inflamed paw in dogs

Score	Definition of the Lameness Score
0	No lameness
1	Barely detectable lameness over most of the observation period
2	Mild lameness, substantial weight bearing
3	Moderate lameness, minimal weight bearing
4	Severe lameness, the animal uses his paw (walking movement initiated and/or touches lightly the ground) but does not bear weight
5	The animal could not be more lame, refuse to move and/or avoid any contact of the inflamed paw with the ground

meloxicam). Two supplementary measures were obtained on the 35th day after test article administration (body temperature and paw circumference) and at 64 days (paw circumference).

End Point Measurements. The six clinical end points selected to assess inflammation were as follows: 1) body temperature (a single measurement was obtained at each time using an electronic thermometer), 2) paw circumference (duplicate measures per time point) measured just above the pad using a measuring tape (DMC, Colmar, France), 3) lameness scored using a numerical rating scale as developed by Giraudel et al. (2005b) (Table 1), 4) creeping time (i.e., the time required by the dog to creep under a 6-m-long tunnel) measured in triplicate at each measurement time and with the mean time computed, 5) vertical normalized force (i.e., the maximal vertical force applied on the ground by a hind limb measured with force plates) (SATEL Vêto; Patrick Savet, Blagnac, France); for each measurement time, three valid measures were recorded and the mean normalized to the dog's body weight ($F_{\max}/b.wt.$, kg/kg) used in the data analyses, and (6) pain as assessed using a hind paw thermal escape model (Hargreaves et al., 1988). The model consisted of exposing the hind limb to a light beam delivered by a Hargreaves apparatus (model 390; IITC Inc., Woodlands Hills, CA) (Giraudel et al., 2005b). This equipment has a light source (halogen 24 V, 150 W) that creates and controls heat intensity. A heat intensity corresponding to 15% of the peak heating value of 150 W was selected. Dogs were placed on a table equipped with a glass top that enabled the beam to be applied from underneath onto a target selected area on the plantar surface of the hind limb. Dogs were trained to stay quiet in control conditions in this environment with minimal manual restraint. When applying the heat stimulus to the top of the metatarsal pad (Fig. 1), a lateral leg movement or any sign of retraction of the paw was considered as a response, and the pain stimulation was stopped immediately. A maximal stimulation time of 90 s was used to prevent any tissue damage through burning. The paw withdrawal time (in seconds) was measured. For each trial, three measures were recorded per measurement time, and the mean of the three measures was used in the data analysis.

Analysis of Meloxicam in Plasma. Plasma samples were analyzed by a high-performance liquid chromatography method using UV detection. In brief, an internal standard (piroxicam) and meloxicam were extracted from the plasma by solid-phase extraction. The high-performance liquid chromatography apparatus consists of a pump system equipped with an automatic injector and an UV detector (360 nm). Separation was achieved by reverse-phase column (150 × 2.0 mm, 3 μm; Diphenyl Pursuit XRs; Varian, Lake Forest, CA) using a guard column (4.0 × 2.0 mm; Phenyl). The mobile phase was a 50:50 mixture of water with 1% acetic acid and acetonitrile (Sigma-Aldrich) at a flow rate of 0.2 ml/min. Under these conditions, meloxicam (Sigma-Aldrich) and piroxicam (Sigma-Aldrich) were eluted at retention times of 7.2 and 5.6 min, respectively. The method was linear over the calibration range from 10 to 1500 ng/ml using a linear model weighted by $1/X^2$. Within-day and day-to-day coefficients of variation were less than 15%, and the accuracy ranged from 101 to 103%, indicating appropriate precision and accuracy for the analytical method. The lack of interference from endogenous

compounds was verified on blank plasma from untreated dogs, establishing the specificity of the method. The validated limit of quantification was 10 ng/ml.

Data Analysis. PK and PK/PD modeling were performed by least-squares regression analysis using WinNonlin Professional software (WinNonlin, version 5.2; Pharsight Corporation, Mountain View, CA).

Individual plasma meloxicam concentrations (ng/ml) were fitted to polyexponential equations. The data points were weighted by the inverse of the squared-fitted value. The number of exponents (two or three) needed to obtain the best fit for each data set was determined by the Akaike's information criterion (Yamaoka et al., 1978) and by inspection of the plot of residuals. On the basis of this approach, a biexponential equation corresponding to a monocompartmental model for extravascular administration with a lag time was selected (eq. 1).

$$C(t) = \frac{FDk_{01}}{V(k_{01} - k_{10})} [\exp(-k_{10} \times (t - t_{lag})) - \exp(-k_{01} \times (t - t_{lag}))] \quad (1)$$

where $C(t)$ is the meloxicam plasma concentration (ng/ml) at time t (h), V/F (l/kg) is the apparent volume of distribution, k_{01} (1/h) is the rate constant of the initial ascending phase, k_{10} (1/h) is the rate constant of the terminal phase, and D is the meloxicam dose (mg/kg). The parameters (V/F , k_{01} , k_{10} , and t_{lag}) were estimated.

For PK/PD modeling, raw measured data were used directly as dependent variables for body temperature, lameness score, and creeping time. Because kaolin decreased the vertical force exerted on the force plates or the paw withdrawal time, these variables were transformed to be expressed as a percentage of reduction (100% is the maximal effect observed after kaolin administration) to use the same PK/PD model for all of end points. Individual PK/PD relationships were described using indirect PD response models (Dayneka et al., 1993). In these models, the measured response (R) is assumed to result from factors controlling either the input or the dissipation of the measured response. Different models were explored, including precursor-dependent indirect response models (Sharma et al., 1998), to capture not only the main effect of meloxicam but also the development of rebound phenomena, which were observed after meloxicam action had ceased. Finally, PD data were described by the following sets of differential equations (eqs. 2–4).

In the control condition (i.e., before kaolin administration), for both periods

$$dR/dt = k_{in} - k_{out}R \quad (2)$$

where dR/dt is the rate of change of the response over time, k_{in} represents the zero-order rate constant for the production of the response, and k_{out} is the first-order rate constant for the loss of the response.

The time development of the kaolin action, noted as $kao(t)$, on the different measured responses was incorporated additively to k_{in} in the model described by eq. 3

$$dR/dt = k_{in} + kao(t) - k_{out}R \quad (3)$$

where $kao(t)$ is the zero-order input rate function (same units as k_{in}) representing the action of the kaolin accounting for the temporal increase in the response after kaolin administration. Such a model already has been selected by other researchers for the PK/PD modeling of the antipyretic effect of naproxen in a model of endotoxin-challenged rats (Josa et al., 2001). After consideration of the inverse U shape of the time development of the responses in the placebo period, $kao(t)$ was modeled using an empirical biexponential equation of the form (eq. 4)

$$kao(t) = P1[\exp(-P2(t - t_{lag})) - \exp(-P3(t - t_{lag}))] \quad (4)$$

with P_1 the intercept (response unit per hour), P_2 (1/h) the slope of the decreasing phase of the kaolin action, P_3 (1/h) the rate constant reflecting the increasing phase of the kaolin action after kaolin administration, and t_{lag} the time of kaolin administration. The three parameters (P_1 , P_2 , and P_3) were estimated simultaneously with the PD parameters of ultimate interest (i.e., k_{in} , IC_{50} , and k_{out} ; see below), and the lag time was fixed at the actual time of kaolin administration for both placebo and test periods.

The effect of meloxicam (melox) was described as the consequence of the inhibition of the phlogogenic effect of kaolin, that is, $kao(t)$ and was incorporated in eq. 3 as (eq. 5)

$$dR/dt = k_{in} + kao(t) \times (1 - \text{melox}) - k_{out} \times R \quad (5)$$

where melox is the classic fractional I_{max} function of the form (eq. 6)

$$\text{Melox} = \frac{I_{max} \times C(t)^{n1}}{IC_{50A}^{n1} + C(t)^{n1}} \quad (6)$$

where melox is a Hill equation in which $C(t)$ (ng/ml) is the meloxicam plasma concentration at time t (h) as obtained by fitting eq. 1, IC_{50A} (ng/ml) is the meloxicam plasma concentration producing half the maximum meloxicam effect (i.e., 50% of I_{max}), I_{max} is a scalar with a maximal value of 1, and $n1$ is the slope of the concentration-effect relationship. For the test period, it was nearly always observed that after the meloxicam action vanished the response increased again (indicating that kaolin action was still present), and unexpectedly, there was an overshoot of the response compared with measures made at the same time in the placebo period. To take into account this second effect of meloxicam on the fate of the kaolin inflammation itself, we rendered time-dependent P_2 , the rate constant reflecting the kaolin inflammation disappearance (see eq. 4), according to eq. 7

$$P2, \text{ melox} = P2 \times \left(1 - \frac{C(t)^{n2}}{IC_{50B}^{n2} + C(t)^{n2}}\right) \quad (7)$$

where $P2, \text{ melox}$ is the time-dependent rate constant reflecting the disappearance of the kaolin action, the value of which was controlled by the plasma meloxicam concentration $[C(t)]$ throughout a fractional I_{max} function with IC_{50B} being the meloxicam plasma concentration halving $P2$. Equation 7 says that $P2, \text{ melox}$ decreases when the plasma meloxicam concentration increases (i.e., meloxicam is delaying the disappearance of the kaolin action, and the natural time course of inflammation resumes when meloxicam is no longer present) (see *Discussion*).

Finally, 10 parameters were estimated by simultaneously fitting the placebo period (with data collected before and after kaolin administration) and the test periods (with data collected before kaolin administration, after kaolin administration but before meloxicam administration, and after meloxicam administration), namely, k_{in} , k_{out} , IC_{50A} for the main anti-inflammatory effect of meloxicam, IC_{50B} for effect of meloxicam on the time development of the kaolin, $n1$, $n2$, I_{max} , and P_1 to P_3 .

Through the use of mean PK and PD parameters, simulations were performed to predict the time development of the response for kaolin alone or kaolin followed by meloxicam for each end point and for doses ranging from 0.05 to 2 mg/kg. Data from all of the individual animals were used to compute the mean PK parameters, but only those dogs with an acceptable PD fit (six to eight animals depending on the PD end points) were taken into account for the calculation of the mean PD parameters.

For each simulation, the maximal response obtained for each response profile was used as a metric to further characterize the dose-response relationship. Then, the dose (mg/kg) versus simulated maximal response was fitted using a classic inhibitory sigmoid model (model of the form of eq. 8)

$$\text{Effect} = \text{Baseline} \times \left(1 - \frac{I_{max} \times \text{Dose}^n}{ED_{50}^n + \text{Dose}^n}\right) \quad (8)$$

where ED_{50} is the dose giving half the maximal response and n is the slope of the dose-response relationship.

To estimate the extent of cyclooxygenase (COX)-1 and COX-2 enzyme inhibition corresponding to the different estimated in vivo IC_{50} values, we computed the percentage of COX-1 and COX-2 inhibition from ex vivo results of a whole-blood assay performed in another group of Beagle dogs. COX-1 activity was determined by measuring serum thromboxane B_2 synthesis in blood samples. COX-2 activity was determined by measuring prostaglandin E_2 synthesis in blood samples incubated at 37°C for 24 h in the presence of lipopolysaccharide. The data were fitted to Hill plots, and the slope (β) and IC_{50} values were calculated. For prostaglandin PGE2 inhibition, β was estimated to be 0.986, and the IC_{50} value was estimated to be 0.1454 μ M (51 ng/ml); corresponding values for thromboxane B_2 were 1.024 and 1.215 μ M (427 ng/ml). Through the use of these parameters kindly provided by Novartis, the I_{max} model was used to estimate the extent of COX-1 and COX-2 inhibition corresponding to our in vivo estimated IC_{50} value.

Statistical Analysis. Metrological performances of the different end points were assessed using the following statistical model (Systat 10; Systat Software, Inc., San Jose, CA)

$$Y_{ijk} = \mu + A_i + D_j + T_k + A * D_{ij} + A * T_{ik} + A * T_{jk} + \epsilon_{ijk}$$

where Y_{ijk} is the end point value for animal i at day j and time k , μ is the overall mean, A_i is the animal factor, D_j is the day factor, T_k is the measurement time factor, $C * D_{ij}$, $C * T_{ik}$, and $D * T_{jk}$ are the corresponding interactions, and ϵ_{ijk} is the residual error for $i = 1$ to 8, $j = 1$ to 5, and $k = 1$ to 3.

All of the variables in this model were considered as fixed factors, and a factor was considered significant when $p < 0.05$.

An analysis of variance was used to test the significance of the different factors and to calculate the repeatability of measurements (as a coefficient of variation) using the residual of the model:

$$\text{Repeatability: CV\%} = \frac{\sqrt{\text{Mean square of residual error}}}{\text{mean}} \times 100$$

Repeatability is the highest level of precision obtained in a given dog for a given day and a given measurement time. Reproducibility (as a coefficient of variation) was calculated based on all of the values recorded during the five days of the study:

$$\text{Reproducibility: CV\%} = \frac{\text{SD}}{\text{mean}} \times 100$$

Reproducibility (always higher than repeatability) is the lowest level of precision encompassing all of the factors of variability in a measurement.

The PK and PD results are presented as mean or median and S.D. For the half-lives, the harmonic mean was calculated. An ANOVA analysis was used to test the significance of the difference between different IC_{50} values.

Results

Metrological Performance of the PD End Points (Repeatability and Reproducibility). The results are summarized in Table 2. The dog factor was highly significant for all of the end points ($p < 0.001$, data not shown), reflecting the interanimal variability and justifying a crossover design to assess the dogs' responses in both placebo and test conditions. For several end points, there were significant animal per day interactions, meaning that the day-to-day variation that could be observed for an end point measurement did not follow a systematic trend as would have happened if a "training" effect had occurred during our validation tests. The measurement time (within day) effect was significant for the

TABLE 2

Repeatability and reproducibility of the selected endpoints
Results are expressed as the coefficient of variation of repeatability and reproducibility (%).

	Repeatability CV	Reproducibility CV
	%	
Body temperature	0.41	0.5
Circumference of the paw	0.44	3.2
Creeping time under a tunnel	4.66	24.2
Vertical normalized maximal force of the paw as obtained with a force plate	3.26	7.1
Paw withdrawal time to a heat stimulus	15.14	20.1

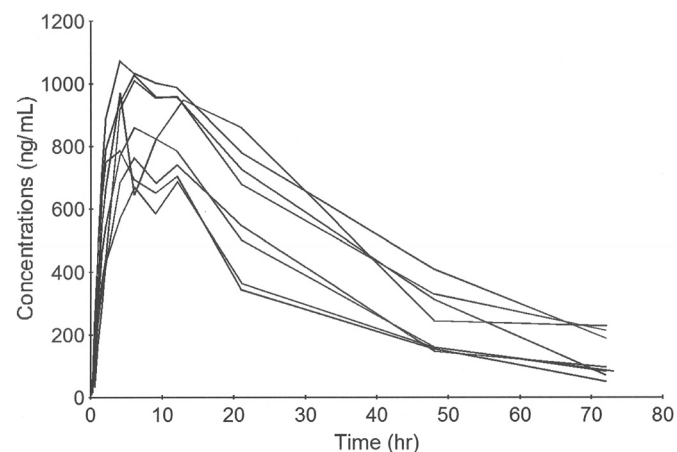


Fig. 2. Spaghetti plot of meloxicam plasma concentration (ng/mL) versus time in eight dogs after a subcutaneous administration of meloxicam (0.2 mg/kg).

body temperature. This observed trend was interpreted as reflecting an endogenous circadian rhythm, the body temperature in the afternoon always being higher than that in the morning. This possible confounding factor was not considered in our PK/PD modeling because the absolute difference associated with this factor was only 0.1°C.

Repeatability (i.e., variability within a given measurement time) was appropriate for all of the end points (<5% for the measures of creeping time and vertical normalized maximal force and <0.5% for measures of body temperature and paw circumference). For the withdrawal time measuring analgesia, the CV of repeatability was 15.1%, which is low considering the expected amplitude of the kaolin and meloxicam effect. Reproducibility (i.e., the overall variability between dogs, days, and measurement times) was <5% for the measures of body temperature and paw circumference, <10% for the measure of the vertical normalized maximal force, and <25% for the creeping time and the paw withdrawal time. The animal factor was the main contributor to the overall reproducibility, thus justifying the use of a crossover design for this kind of investigation rather than a parallel design.

PK Results. The meloxicam plasma concentration profile after subcutaneous administration of 0.2 mg/kg was interpreted as a monocompartmental model with a first-order rate constant of absorption and a lag time (Fig. 2). The apparent total plasma clearance (Cl/F , clearance scaled by the unknown bioavailability) was low ($6.33 \pm 1.95 \text{ ml} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$), and the apparent volume of distribution (V/F , volume of distribution scaled by the unknown bioavailability) was

small $192.1 \pm 34.4 \text{ ml/kg}$. Peak meloxicam plasma concentration ($890.2 \pm 166.4 \text{ ng/ml}$) was attained at $5.97 \pm 1.73 \text{ h}$ after administration, and the harmonic mean of the apparent half-life of absorption was $1.32 \pm 0.59 \text{ h}$. The harmonic mean of the terminal half-life was relatively high at $21.26 \pm 4.83 \text{ h}$.

Time Development of the Kaolin Paw Inflammation Model with and without Meloxicam Administration.

Figure 3 illustrates the time course and magnitude of the inflammatory response for the six investigated end points in both placebo and treated conditions. The end point values reached maxima 22 and 24 h after the kaolin injection. For the placebo period (without meloxicam), body temperature, creeping time, and paw withdrawal time still were altered 56 h after kaolin administration (spontaneous improvement was <50%, a 100% improvement meaning return to the prekaolin administration condition). Paw edema (measured by the paw circumference) disappeared slowly, a delay of 15 days being necessary to reduce the kaolin-induced edema by approximately 50%. The vertical force exerted by the hind limb still was decreased by approximately 50% on the 10th day after the kaolin administration, indicating a rather sustained inflammation that enables NSAID effects to be followed over a prolonged time period. However, the natural time course of the inflammation was not stable enough to be ignored when modeling the NSAID effect, and a crossover design including a placebo period was mandatory to assess the net effect of the NSAID (see *Discussion*). In terms of animal welfare, it was observed that for the six investigated end points an improvement superior to 85% was observed after kaolin administration between the 14th and 17th days (body temperature), the 24th and 35th days (paw circumference), the 17th and 21st days (lameness score), the 10th and 14th days (creeping time), the 10th and 14th days (vertical force of the paw exerted on the ground), and the 7th and 10th days for the allodynia as assessed by paw withdrawal time.

In treated conditions (after a subcutaneous injection of meloxicam at a dose of 0.2 mg/kg), a clear-cut drug response was obtained for all but one end point, namely, paw circumference. The unresponsiveness of paw circumference to meloxicam was expected (see *Discussion*) and was not considered for further analysis. The mean time of the peak response occurred for all of the end points between 4.6 and 7.3 h after meloxicam administration. The mean time of the maximum decrease in body temperature was slightly shorter (4.6 h) than the mean time of the peak response for other end points. The mean paw withdrawal time obtained 6 h after meloxicam administration ($16.54 \pm 6.44 \text{ s}$) was similar to the mean baseline withdrawal time without inflammation ($16.79 \pm 3.03 \text{ s}$), indicating that complete suppression of allodynia was achieved with 0.2 mg/kg meloxicam. The body temperature, lameness score, creeping time, and vertical force of the paw values did not return to baseline values, the maximal percentages of improvement being of 70, 59, 79, and 66%, respectively. An average duration of drug response was observed for approximately 20 h for all of the end points. After completion of the meloxicam action, the time course of the inflammation did not return to the one observed at the same time during the placebo period, but rather there was for all of the measured end points an overshoot from the 21st hour after meloxicam administration and the following 2 or 3 days.

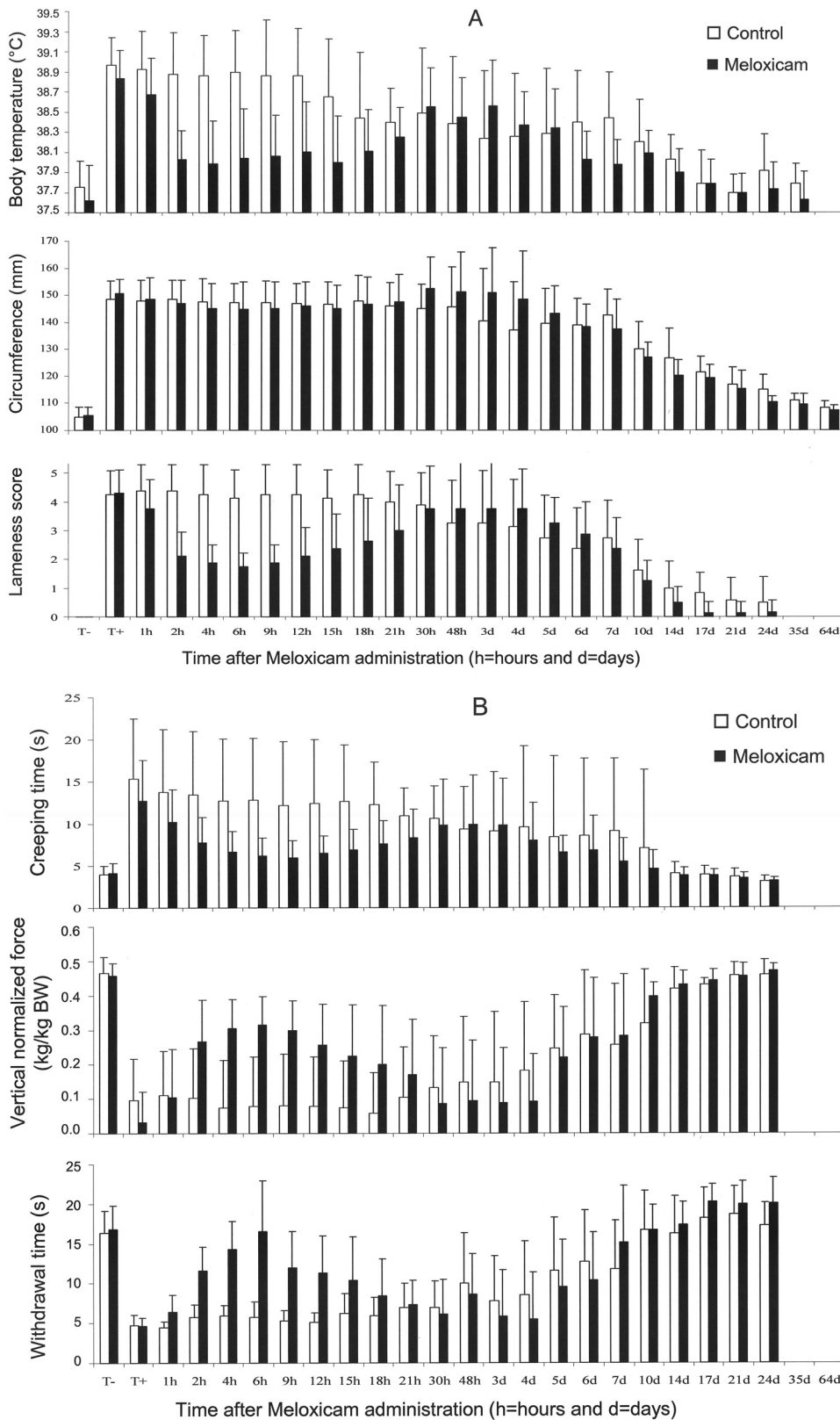


Fig. 3. Time course of six end points (mean \pm S.D.). A, body temperature, paw circumference, and lameness score. B, creeping time and vertical normalized force of the paw and pain expressed as withdrawal time of the paw. Data obtained in eight dogs in treated (meloxicam, 0.2 mg/kg) and placebo conditions after an injection of 1.85 g of kaolin in the hind paw. T- are control values obtained before kaolin administration; T+ are control values obtained after administration of kaolin but before meloxicam or placebo administration.

PK/PD Analysis. Figure 4 shows the fit of the responses for all of the end points (body temperature, lameness score, creeping time, vertical force of the paw on the ground, and paw withdrawal time) for a representative dog. For the eight dogs investigated and the five measured end points, a successful fit was obtained for 34 of the 40 recorded time courses.

Table 3 gives the mean PD parameters for all of the end points. Mean values of IC_{50A} for the meloxicam effect on $kao(t)$ ranged from 210 ng/ml for body temperature to 546 ng/ml for the vertical force measured by the force plate. The IC_{50A} value for body temperature was lower than the other IC_{50A} values, but the differences were not significant

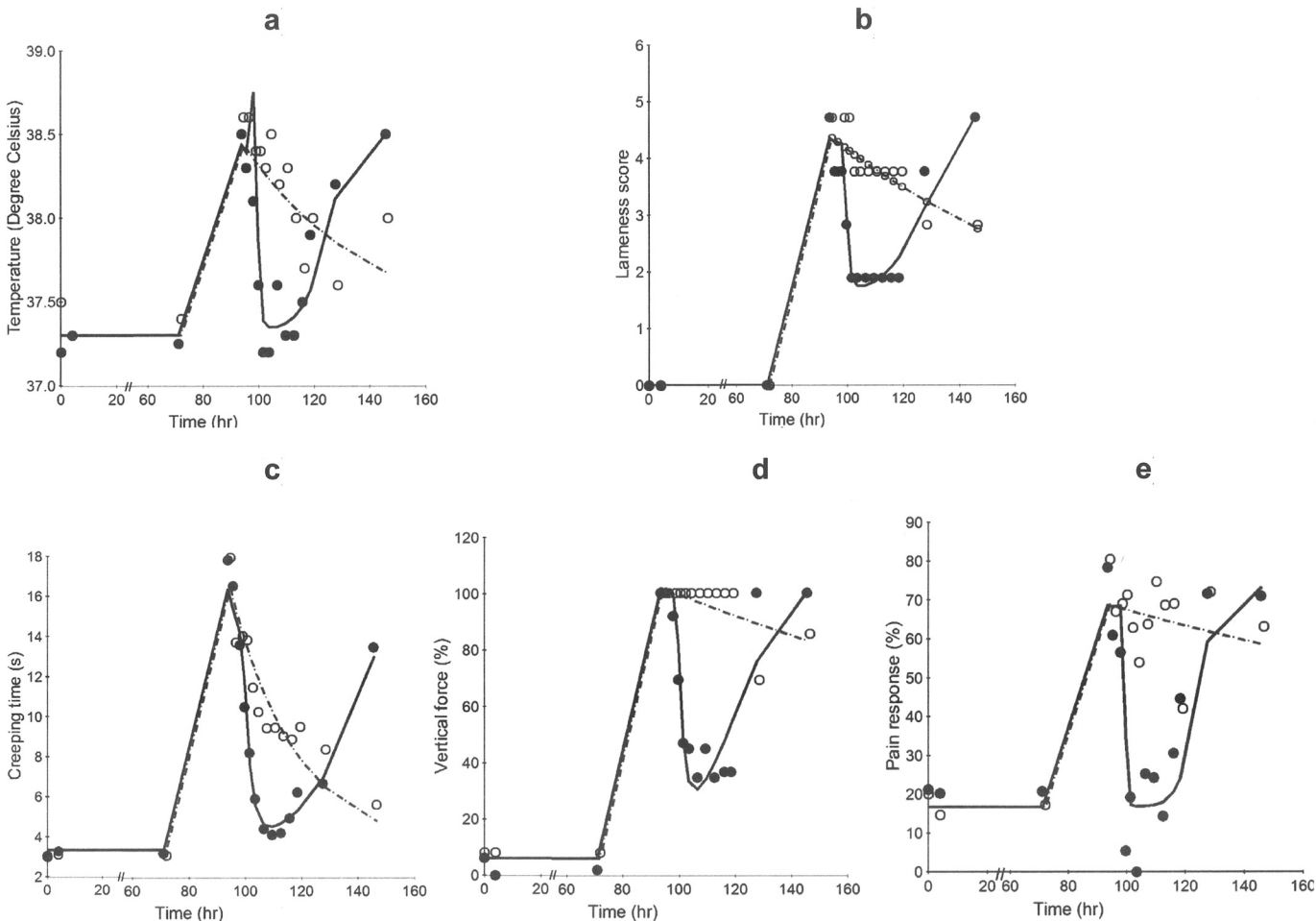


Fig. 4. Observed (●, ○) and fitted values during the placebo (○, - - -) and the test period (●, —) of the five investigated end points in a representative dog after a single subcutaneous meloxicam administration at a concentration of 0.2 mg/kg. A–E, body temperature (°C) (A), lameness score (B), creeping time (s) (C), vertical force exerted by the paw expressed as a percentage of the maximal observed response to kaolin to a heat stimulus during the placebo and the treated (meloxicam, 0.2 mg/kg) period (E). Kaolin was administered at 71 and 71.967 h during the test and placebo period, respectively; meloxicam was administered at 97.5 h.

TABLE 3

Estimated mean pharmacodynamic parameters describing meloxicam anti-inflammatory, analgesic, and antipyretic effects after a subcutaneous administration of meloxicam at a dose of 0.2 mg/kg to eight dogs

Units			Definition		Body Temperature (CV%)		Lameness Score (CV%)		Creeping (CV%)		Force Plate (CV%)		Analgesia (CV%)	
k_{in}	Response per hour		Zero-order rate constant for production of the response		94.6	(14)	0.0057	(25)	13.8	(57)	7.64	(119)	43	(63)
IC_{50B}	Nanograms per milliliter		Plasma meloxicam IC_{50} on the time development of kaolin inflammation		69	(51)	56	(28)	72	(27)	68	(37)	61	(28)
$n2$	No unit		Hill coefficient associated with IC_{50A}		2.61	(51)	1.87	(23)	2.91	(83)	2.27	(74)	1.13	(77)
k_{out}	h^{-1}		First-order rate constant for loss of response		2.51	(14)	4.99	(49)	3.45	(63)	3.03	(123)	5.37	(45)
I_{maxA}	No unit		Maximal possible clinical effect (between 0 and 1)		0.84	(11)	0.82	(16)	1	(N.A.)	1	(N.A.)	1	(N.A.)
IC_{50A}	Nanograms per milliliter		Plasma meloxicam concentration for $I_{maxA}/2$ for the clinical endpoints		210	(64)	466	(55)	400	(86)	546	(64)	390	(65)
$n1$	No unit		Hill coefficient associated with IC_{50B}		4.37	(66)	3.93	(85)	2.56	(91)	3.03	(95)	3.88	(105)
P_1	Same as k_{in}		Parameters characterizing time development of kaolin inflammatory		5.10	(35)	24.3	(46)	32.8	(42)	303	(125)	483	(56)
P_2	h^{-1}		action (see eq. 4)		0.023	(47)	0.0038	(100)	0.015	(86)	0.0052	(130)	0.0112	(101)
P_3	h^{-1}				0.96	(20)	1.05	(10)	0.94	(16)	0.71	(72)	0.95	(21)

N.A., not applicable.

(analysis of variance, $p > 0.05$). The interanimal variability of the estimated IC_{50A} value was estimated using a coefficient of variation that ranged from 28 (for analgesia) to 86% (creeping time) with intermediary values for the other end points.

The meloxicam sensitivity (i.e., the slope of the concentration-effect relationship) was relatively high for all of the end points (from 2.56 to 4.37), indicating that the effect was rapidly reaching I_{max} with increasing meloxicam plasma concentrations.

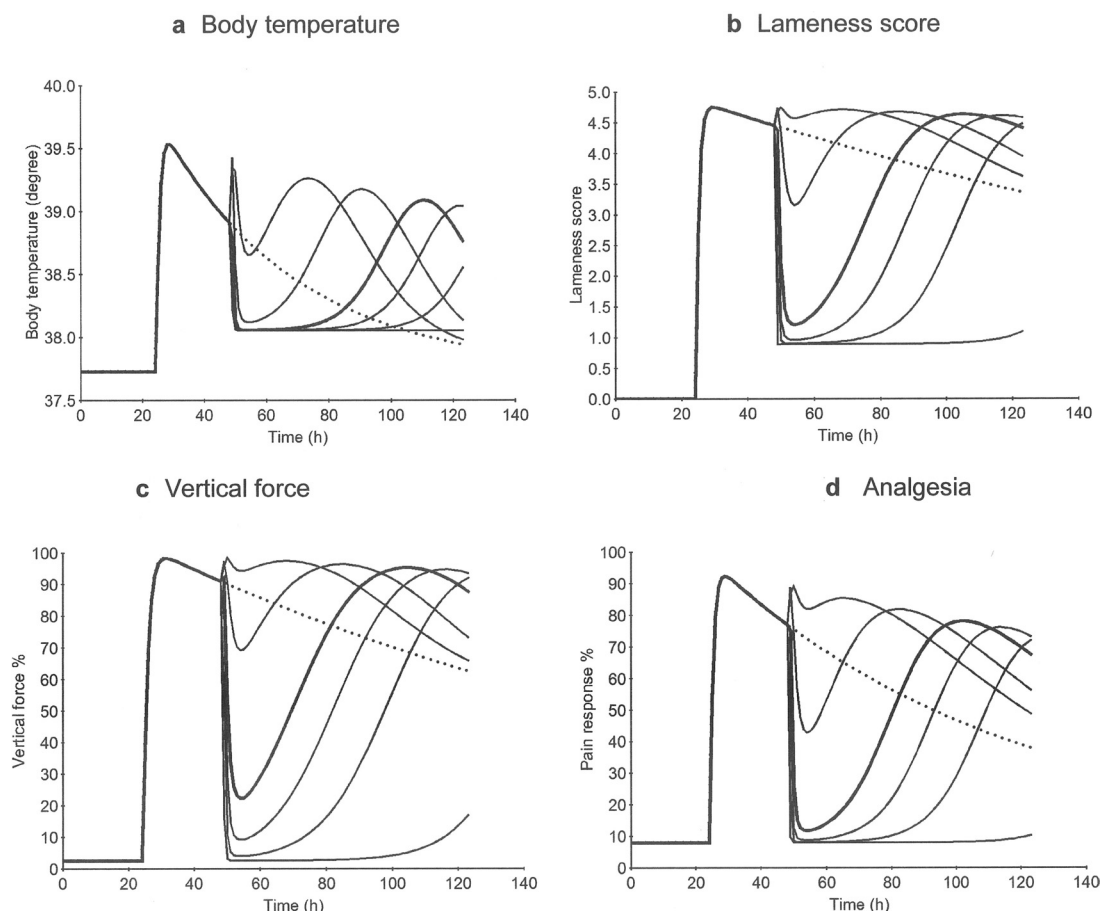


Fig. 5. Simulated values of four investigated end points after a single subcutaneous meloxicam administration at 0 (placebo), 0.055, 0.1, 0.2 (thick line), 0.3, 0.5, and 2 mg/kg b.wt. A–D, body temperature (°C) (A), lameness score (B), vertical force exerted by the paw expressed as a percentage of the maximal observed response to kaolin (C), and paw withdrawal time expressed as a percentage of the maximal observed response to kaolin to a heat stimulus (D). Kaolin and meloxicam were administered at 24 and 48 h, respectively. The presence of a rebound (compared with the placebo period) is clearly seen for the four end points.

In the current modeling, meloxicam developed a second action through a modulation of $k_{ao}(t)$. The estimated IC_{50B} values for this secondary effect were systematically lower than the corresponding IC_{50A} values of the main effect of meloxicam [mean IC_{50A}/IC_{50B} ratio from 6 (for analgesia) to 8 (for vertical forces)]. The IC_{50B} value expressed the potency of meloxicam to delay the disappearance of kaolin inflammation, whereas the IC_{50A} value expressed the potency of meloxicam to mitigate the clinical expression of the inflammation (see *Discussion*).

Mean PK and PD parameters were used to simulate meloxicam dosage regimens ranging from 0.05 to 2 mg/kg for body temperature, lameness score, vertical force on a force plate, and paw withdrawal time (Fig. 5). Considering the maximal simulated responses, the dose-response relationships were modeled (Fig. 6), and the estimated ED_{50} values were calculated for the different end points. The ED_{50} value was 0.0507 mg/kg for body temperature and from 0.091 to 0.127 mg/kg for the other end points.

The whole-blood ex vivo assay is useful for predicting the clinical relevance of given levels of COX-1 and COX-2 inhibition (Fig. 7). For the present experiment, it was calculated that the plasma concentration corresponding to our estimated in vivo IC_{50A} value would ensure ex vivo an inhibition from approximately 80 (body temperature) to 90% (others

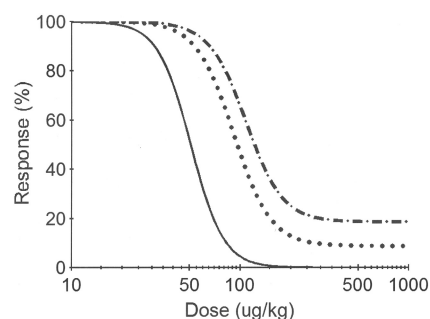


Fig. 6. Dose-response relationships for meloxicam. The effects [body temperature (—), withdrawal time (•••••) of the paw as a measure of analgesia, and lameness score (—•—•—)] were simulated with the PK/PD model using mean PK and PD parameters; the relationship between the dose of meloxicam (mg/kg) and the maximal response obtained for each dose were fitted with an inhibitory sigmoidal model (see eq. 8 in the text).

end points) of the COX-2 isoenzyme and from 32.6 to 52.9% of the COX-1 isoenzyme (Table 4). The in vivo COX-2 versus COX-1 selectivity, as evaluated by the ratio of COX-2/COX-1 inhibition for a plasma concentration corresponding to the in vivo IC_{50A} value, was from 1.72 to 2.46 depending on the end point. The in vitro IC_{50} value (51 ng/ml) versus the in vivo IC_{50A} ratio was from 4.1 to 10.7 depending on the end point.

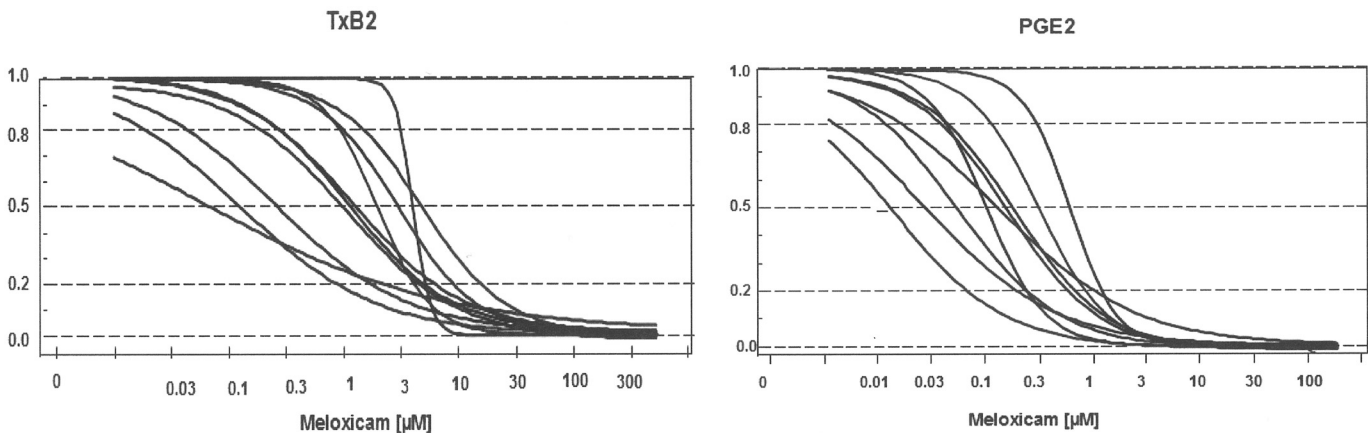


Fig. 7. Ex vivo COX-1 and COX-2 inhibition in nine dogs. Relative change (0–1) against \log_{10} blood concentrations of meloxicam in nine dogs of thromboxane B_2 as an index of COX-1 inhibition and prostaglandin E_2 as an index of COX-2 inhibition (Novartis file; for details, see King et al., 2010).

TABLE 4
Extent of COX-1 and COX-2 enzyme inhibition (from 0 to 100%) corresponding to the different estimated in vivo IC_{50A} values
The in vivo versus in vitro potency ratio was obtained by dividing the in vivo IC_{50A} value (see Table 3) by the ex vivo IC_{50} value obtained from the whole-blood assay [i.e., 51 ng/ml (0.1454 μ M)].

Parameters	%COX-2 Inhibition	%COX-1 Inhibition	In Vivo Selectivity	Ex Vivo $IC_{50}/$ In Vivo IC_{50A} Ratio
Body temperature	80.1	32.6	2.46	4.1
Lameness score	89.8	52.2	1.72	9.1
Creeping time	88.4	48.3	1.83	7.8
Force plates	91.2	52.9	1.73	10.7
Analgesia	88.1	47.7	1.85	7.6

Discussion

The main motivation of the present study was to develop a humane inflammation model for preclinical PK/PD investigations in dogs. The dog is a standard species for the preclinical development of drugs, and its use should be ethically acceptable. In a European Union context, this feature for an animal model is now critically scrutinized, and a fully reversible inflammatory model is considered as desirable because it allows dogs used in this type of trial to be reallocated after the completion of the study to other protocols or, in our case, to be definitively rehabilitated as companion animals. Kaolin was selected as a phlogistic agent because it acts mainly as an inert foreign body. In rats, kaolin does not activate the complement cascade (Noordhoek et al., 1977) but rather triggers the release of kinins and prostaglandins (Lewis, 1978). When administered outside an articulation, it is eliminable through sterile abscess formation, an inflammation resolution that guarantees a lack of delayed side effects. This is not the case for inflammation models having an immunological component such as the classic Freund adjuvant arthritis model that leads to the development of an irreversible osteoarthritis in dogs. In addition, we recently showed that the subcutaneous injection of kaolin into a cat's paw produced a well defined, reproducible, and reversible inflammatory response (Giraudel et al., 2005b), and this model was found to be suitable for studying simultaneously the analgesic, anti-inflammatory, and antipyretic effects of NSAIDs.

From the present trials, it can be concluded that the kaolin model in dogs is ethically acceptable. It mimics the natural process of abscess formation and maturation, and it was fully

reversible within 8 weeks. We now use this model routinely, and we rarely observe side effects such as paw infection (one case for 40 dogs), but limited skin necrosis is a more frequent finding in this model.

To be useful for preclinical investigations of NSAIDs, a canine inflammatory model should be able to predict the order of magnitude of a future dosage regimen in different target species including man. To assess this property, we selected meloxicam as a test NSAID because it has been used extensively as a therapeutic agent in both animals and humans and its dosage regimen is now well established in both man and dogs (Engelhardt, 1996; Türck et al., 1996; Busch et al., 1998; Slingsby and Waterman-Pearson, 2000; Lascelles et al., 2001). In addition, the PK profile of meloxicam was known to be very similar in man (Davies and Skjodt, 1999) and dogs (Busch et al., 1998). This has been confirmed in the present study where the plasma clearance determined in dogs (Cl/F , 6.3 ml/kg per hour) can be considered as practically equal to the clearance after intravenous administration in man (6.1 ml/kg per hour for a body weight of 70 kg) (Türck et al., 1996). Only one meloxicam dose was investigated due to our two-way crossover design for two hind legs with a control and a test period, but the selected dose was likely high enough to sweep all of the concentration-effect relationship, allowing us to properly estimate PD parameters.

The main limit of this model is the lack of a prolonged steady inflammation over several days to enable a NSAID to be tested on a stable inflammation baseline. Indeed, the price to pay for a reversible inflammatory model is to have inflammation with a rapid time course, increasing over 2 to 3 days and then decreasing immediately over the next few days (i.e., without a well defined plateau). The practical consequence of that is the necessity to characterize precisely the time development of the inflammation progression during a placebo period for each tested dog and to model simultaneously the placebo and the test period to take into account the confounding factor of the natural time development of kaolin inflammation. This is what we have carried out with the present modeling approach. Several models of disease progression were proposed (for an extensive review, see Mould, 2007). In this study, we selected a simple Bateman function describing the transient effect of kaolin. In fact, the time course of the kaolin inflammation was not the same during the placebo and test periods. For several end points, we observed a re-

bound effect (i.e., that after cessation of meloxicam action the levels of paw inflammation were greater than those obtained at the same time after placebo dosing). A possible explanation for this delaying action of meloxicam on the disappearance of the inflammation is the classically reported impeding action of NSAIDs on abscess formation that is required to physically eliminate the administered kaolin. Therefore, we took into account in our mathematical model this effect of meloxicam on the progression of the inflammation by modulating the actual value of P_2 (see eq. 4), the parameter reflecting the rate of inflammation disappearance. P_2 was modulated by the actual meloxicam concentration through a Hill inhibitory function (see eq. 7). This part of our model predicted that for a high meloxicam plasma concentration the time development of the kaolin action was slowed down temporarily or even stopped. It should be noted that our model is not equivalent to classic paw edema as observed in rats after the intra-articular administration of a phlogistic agent. In our case, the increase in the paw volume was associated with lymph node hypertrophy (popliteal), which can be interpreted as a lymphatic drainage blockade protecting the animal from systemic exposure to kaolin particles. This explains why meloxicam (as other NSAIDs) has no short-term effect on the paw diameter. As an alternative to this model, we also explored a precursor-dependent indirect PD response model, as outlined by Sharma et al. (1998), to describe tolerance and rebound phenomena. This model fit our data well and also was able to capture adequately the rebound phenomena. However, it was associated with generally poor precision in the individual estimates of IC_{50} values, and this was probably due to a problem of numerical identifiability. It is possible that investigating several dose rates with a population modeling approach might have rendered this kind of PK/PD model usable, because only a single dose could be tested for each dog based on our crossover design with two periods (placebo and test article) corresponding to the two hind limbs. This is a possible limitation to this paw model, because studying a single dose rate for a NSAID may lead to some estimation difficulties for PD parameters. This would arise especially if the tested dose was too low. Using our PK/PD model, we estimated the meloxicam potency for different end points of clinical relevance, and we also confirmed the general statement that potency is usually similar between species (Levy, 1993; Busch et al., 1998), because the present results are very similar to those obtained in cats using the same model (Giraudel et al., 2005a) and in man (Davies and Skjodt, 1999). Because the meloxicam kinetic parameters (clearance and half-life) and meloxicam potency are very similar in dogs and man, the present canine model should be able to predict a relevant dosage regimen in man. In this study, simulations for the different end points using the PK/PD model for different dose levels allowed us to estimate an ED_{50} value of 0.05 mg/kg for an antipyretic effect, an ED_{50} value of 0.09 for analgesia, and an ED_{50} value of 0.127 mg/kg for the locomotion end points (vertical force assessed by force plate). These doses are very similar to the current clinically recommended dose not only in dogs (0.1 or 0.2 mg/kg) but also in humans (7.5–15 mg, i.e., between 0.1 and 0.3 mg/kg for a body weight of 50–70 kg). This comparison demonstrates the potential usefulness of this preclinical PK/PD modeling approach for predicting a dosage regimen, not only in the dog as a target species but also in other species including man. In

both instances, well designed clinical studies will be needed to confirm or adapt the dosage regimen to clinical conditions that might be less severe than those in this acute paw inflammation model.

Because the in vivo determined potencies calculated in the present study are likely to reflect meloxicam effects on prostaglandin synthesis, it is relevant to compare these in vivo IC_{50A} values with the potency for COX-2 inhibition obtained in vitro or ex vivo with the whole-blood assay. We showed that the plasma concentration corresponding to our estimated in vivo IC_{50A} value would ensure, in a canine ex vivo whole-blood assay, an inhibition of approximately 80% for body temperature and 90% for the other end points of the COX-2 isoenzyme. These results are consistent with what we observed for several other NSAIDs (i.e., that an inhibition close to 90% of the COX-2 isoenzyme is predictive of clinical efficacy) (Toutain, 2002). Thus, the kaolin model could be relevant for in vitro (ex vivo) to in vivo extrapolations.

In conclusion, we have developed an ethical dog inflammation model and using meloxicam as a reference NSAID. We showed that this model would be useful to investigate dose-effect relationships of NSAIDs and also could be relevant for in vitro to in vivo extrapolations. The limitation of the model is the requirement of a rather advanced PK/PD analysis to accurately estimate the PD parameters of interest.

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Authorship Contributions

Participated in research design: Jeunesse, C. Toutain, Letellier, Giraudel, and P. Toutain.

Conducted experiments: Jeunesse, Bargues, and C. Toutain.

Contributed new reagents or analytic tools: Lacroix.

Performed data analysis: Jeunesse and P. Toutain.

Wrote or contributed to the writing of the manuscript: Jeunesse, Letellier, and P. Toutain.

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